

# Macrophage Mal1 Deficiency Suppresses Atherosclerosis in Low-Density Lipoprotein Receptor–Null Mice by Activating Peroxisome Proliferator-Activated Receptor- $\gamma$ –Regulated Genes

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**Objective**—The adipocyte/macrophage fatty acid-binding proteins aP2 (FABP4) and Mal1 (FABP5) are intracellular lipid chaperones that modulate systemic glucose metabolism, insulin sensitivity, and atherosclerosis. Combined deficiency of aP2 and Mal1 has been shown to reduce the development of atherosclerosis, but the independent role of macrophage Mal1 expression in atherogenesis remains unclear.

**Methods and Results**—We transplanted wild-type (WT), Mal1<sup>−/−</sup>, or aP2<sup>−/−</sup> bone marrow into low-density lipoprotein receptor–null (LDLR<sup>−/−</sup>) mice and fed them a Western diet for 8 weeks. Mal1<sup>−/−</sup>→LDLR<sup>−/−</sup> mice had significantly reduced (36%) atherosclerosis in the proximal aorta compared with control WT→LDLR<sup>−/−</sup> mice. Interestingly, peritoneal macrophages isolated from Mal1-deficient mice displayed increased peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) activity and upregulation of a PPAR $\gamma$ -related cholesterol trafficking gene, CD36. Mal1<sup>−/−</sup> macrophages showed suppression of inflammatory genes, such as COX2 and interleukin 6. Mal1<sup>−/−</sup>→LDLR<sup>−/−</sup> mice had significantly decreased macrophage numbers in the aortic atherosclerotic lesions compared with WT→LDLR<sup>−/−</sup> mice, suggesting that monocyte recruitment may be impaired. Indeed, blood monocytes isolated from Mal1<sup>−/−</sup>→LDLR<sup>−/−</sup> mice on a high-fat diet had decreased CC chemokine receptor 2 gene and protein expression levels compared with WT monocytes.

**Conclusion**—Taken together, our results demonstrate that Mal1 plays a proatherogenic role by suppressing PPAR $\gamma$  activity, which increases expression of CC chemokine receptor 2 by monocytes, promoting their recruitment to atherosclerotic lesions. (*Arterioscler Thromb Vasc Biol.* 2011;31:1283–1290.)

**Key Words:** CCR2 ■ CD36 ■ PPAR $\gamma$  ■ macrophages

Fatty acid-binding proteins (FABPs) play important roles in fatty acid transport, cellular signaling, gene transcription, and cytoprotection.<sup>1</sup> FABPs belong to a family of 14- to 15-kDa proteins that bind with high affinity to hydrophobic ligands, such as saturated and unsaturated long-chain fatty acids, eicosanoids, and other lipids.<sup>2</sup> The adipocyte/macrophage FABPs aP2 (FABP4) and Mal1 (FABP5) are intracellular lipid chaperones that modulate systemic metabolism of glucose and lipids, insulin sensitivity, and atherosclerosis.<sup>2</sup> We have previously demonstrated that either deficiency of aP2 or combined deficiency of the aP2 and Mal1 genes significantly attenuates atherosclerosis in apolipoprotein E (apoE)<sup>−/−</sup> mice on a normal chow diet or a high-fat diet.<sup>3–5</sup> Bone marrow transplantation studies demonstrated that the antiatherogenic effect of aP2 deficiency is predominantly, if

not entirely, related to its actions in the macrophage and is independent of the impact of aP2 on insulin sensitivity.<sup>2,5</sup> However, the independent role of macrophage Mal1 expression in atherogenesis has not been studied yet.

Previous reports have shown that macrophage aP2 deficiency significantly enhances the nuclear hormone peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) activity in macrophages, increasing both CD36-mediated uptake of oxidized low-density lipoprotein (OxLDL) and ABCA1-mediated cholesterol efflux in the cells.<sup>6</sup> In addition, aP2<sup>−/−</sup> macrophages have reduced I $\kappa$ B kinase activity and nuclear factor- $\kappa$ B (NF- $\kappa$ B)-related inflammatory gene expression.<sup>6</sup> These aP2-related changes in macrophage cholesterol trafficking and inflammation have a dramatic impact on the development of atherosclerosis. Macrophages express the aP2

Received on: January 19, 2009; final version accepted on: March 16, 2011.

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*Arterioscler Thromb Vasc Biol* is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.111.225839

and Mal1 FABP isoforms at a ratio of approximately 1:1.<sup>5</sup> These 2 proteins have 52% amino-acid similarity and bind various fatty acids and synthetic compounds with similar selectivity and affinity.<sup>7</sup> The high degree of homology in structure and ligand affinity between aP2 and Mal1 suggests that Mal1 may have similar, and possibly redundant, roles to aP2 in macrophage biology and atherogenesis.

Recent studies demonstrated that FABPs act as chaperones, facilitating transport of fatty acids from the plasma membrane to different intracellular compartments.<sup>2</sup> Mal1 expression modulates systemic insulin sensitivity in 2 models of obesity and insulin resistance.<sup>2,8</sup> This may induce basal and insulin-stimulated phosphorylation of Akt in adipose and muscle tissues specific for aP2<sup>-/-</sup>/Mal1<sup>-/-</sup> mice.<sup>9</sup> Akt is a key regulator of macrophage survival and inflammatory responses, and several studies have indicated an important role for macrophage Akt signaling in atherosclerosis.<sup>10,11</sup> However, the impact of Mal1 expression on macrophage Akt expression and the development of atherosclerosis has not been previously examined.

To study the role of macrophage Mal1 in early atherosclerosis, we generated chimeric low-density lipoprotein receptor-null (LDLR<sup>-/-</sup>) mice with Mal1-deficient hematopoietic cells and challenged them with a Western diet for 8 weeks. Recipient mice reconstituted with Mal1<sup>-/-</sup> marrow had significantly smaller (36%) atherosclerotic lesions compared with control mice transplanted with WT marrow. In addition, Mal1<sup>-/-</sup> macrophages displayed a significant increase in PPAR $\gamma$  activity and affected expression of the PPAR $\gamma$ -regulated gene CD36 and genes involved in inflammation, including suppression of CC chemokine receptor 2 (CCR2) levels in monocytes, which likely reduces their recruitment to atherosclerotic lesions.

## Methods

### Animal Procedures

The Mal1-deficient mice were developed using homologous recombination in embryonic stem cells, as described,<sup>12</sup> and backcrossed 10 or more generations onto C57BL/6 background.<sup>13</sup> All recipient LDLR<sup>-/-</sup> mice and corresponding wild-type (WT) controls were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained in microisolator cages on a rodent chow diet containing 4.5% fat (PMI 5010, St. Louis, MO) or on a Western-type diet containing 21% milk fat and 0.15% cholesterol (Teklad, Madison, WI). Animal care and experimental procedures were performed according to the regulations of Vanderbilt University's Animal Care Committee.

### Genotyping and Bone Marrow Transplantation

To identify the Mal1 genotype, we generated a set of primers (GAC GAT ATA AGC GCA GAT GG, AAC TGA GGG GCT GTT TGT AG and TCG CCT TCT ATC GCC TTC TTG AC) producing a 610-bp band specific for the Mal1 targeted allele and a 435-bp band specific for the WT allele by polymerase chain reaction (PCR) analysis. Recipient 8-week-old female LDLR<sup>-/-</sup> mice were lethally irradiated (9 Gy) from a cesium gamma source and transplanted with  $5 \times 10^6$  bone marrow cells from female Mal1<sup>-/-</sup>, aP2<sup>-/-</sup>, or WT donor mice as described.<sup>14</sup>

### Serum Lipids and Lipoprotein Distribution Analyses

Mice were fasted for 4 hours, and then serum total cholesterol and triglycerides were measured by enzymatic methods using reagents

from Raichem (San Diego, CA) and SoftMax Pro5 software (Molecular Devices). Fast performance liquid chromatography was performed on a high-performance liquid chromatography system (model 600, Waters, Milford, MA) using a Superose 6 column (Pharmacia, Piscataway, NJ).

### Analysis of Aortic Lesions

Aortas were flushed through the left ventricle, and cryosections of the proximal aorta were analyzed using the Imaging System KS 300 (Kontron Elektronik GmbH) as described.<sup>15</sup>

### Peritoneal Macrophages: Isolation and Treatment

Thioglycollate-elicited peritoneal macrophages were isolated from WT and Mal1<sup>-/-</sup> mice. Macrophages were treated with 0.5 mmol/L palmitic acid complexed to bovine serum albumin (BSA) as described,<sup>16</sup> with human OxLDL (100  $\mu$ g/mL) or acetylated low-density lipoprotein (AcLDL, 100  $\mu$ g/mL, Intracel Corp, Rockville, MD) plus an ACAT inhibitor, Sandoz 58035 (10  $\mu$ g/mL; Sigma) as described,<sup>17</sup> or with PPAR $\gamma$  agonist, ciglitazone (Cayman Chemicals) or PPAR $\gamma$  antagonist, GW9662 (Sigma).

### Modified Low-Density Lipoprotein Uptake

Macrophages were incubated with 3,3'-dioctadecylindocarbocyanine-labeled human AcLDL or OxLDL (Intracel) at 37°C for 2 hours and analyzed under a fluorescent microscope or by fluorescence-activated cell sorting (FACS) flow cytometry as described.<sup>15</sup>

### RNA Isolation and Real-Time PCR

Total RNA was isolated from macrophages using a Trizol reagent (Life Technologies, Inc) and purified by the RNeasy kit (Qiagen, Valencia, CA). Relative quantitation of the target mRNA was performed using primers, probes, and the Sequence Detection System (Applied Biosystems) and normalized with 18S ribosomal RNA as described.<sup>18</sup>

### Blood Monocyte Analyses

Blood was collected from mice in the presence of 5 units of heparin, and the opaque layer of mononuclear cells was isolated by Histopaque-1077 (Sigma) gradient. Then, cells were kept in a 6-well plate at 37°C for 30 minutes and washed with PBS. CCR2 protein expression was detected by a rabbit monoclonal antibody to CCR2 (Epitomics, Burlingame, CA) and analyzed by FACS (Becton Dickinson) as described.<sup>15</sup>

### Western Blotting

Cells were treated with a cell lysis buffer (Cell Signaling Technology, Danvers, MA) with a protease (Sigma-Aldrich) and a phosphatase inhibitor (Pierce) cocktail. Proteins (20 to 100  $\mu$ g/lane) were resolved by NuPAGE Bis-Tris electrophoresis (Invitrogen) and transferred onto polyvinylidene difluoride nitrocellulose membranes (Amersham Bioscience). Blots were probed with rabbit antibodies to PPAR $\gamma$  (catalog no. ab27649, Abcam, Inc, Cambridge, MA); Akt and p-Akt (from Cell Signaling Technology); c-Rel, NF- $\kappa$ B, p65, and I $\kappa$ B $\alpha$  (Santa Cruz Biotechnology);  $\beta$ -actin (Abcam); and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Upstate Cell Signaling, Lake Placid, NY). Peroxidase enzyme visualized with ECL Western blotting detection reagents (GE Healthcare) on x-ray films. To quantify the bands obtained via Western blot analysis, we used National Institutes of Health ImageJ software (<http://rsb.info.nih.gov/ij/>).

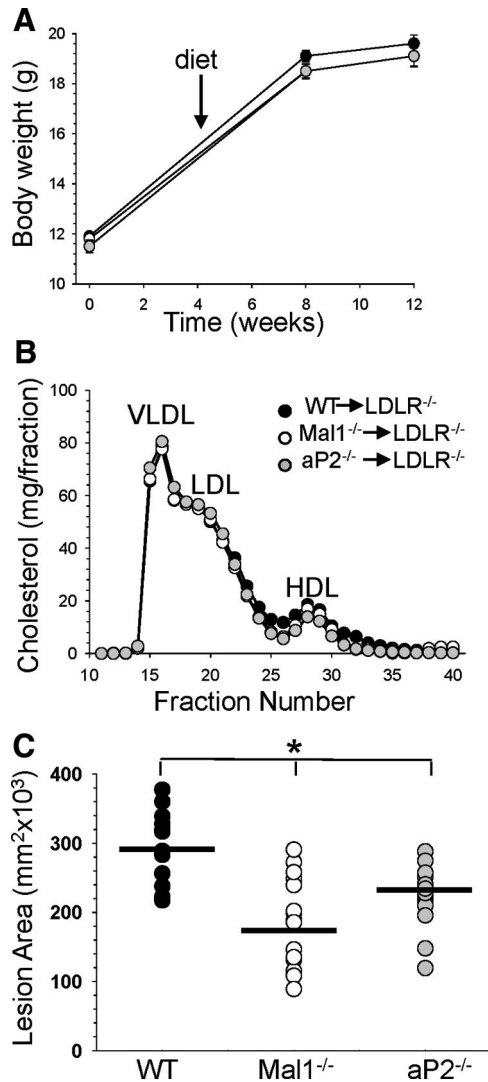
### Statistical Analysis

The statistical differences in mean serum lipids and aortic lesion areas between the groups were determined by 1-way ANOVA test and *t* test.

## Results

### Mal1 Deficiency in Hematopoietic Cells Does Not Affect Serum Lipid Levels but Suppresses Early Atherosclerosis

To study the impact of macrophage Mal1 expression on early atherosclerosis, we used bone marrow transplantation to



**Figure 1.** Changes in body weight (A), serum lipoprotein profiles (B), and atherosclerotic lesion area in the proximal aorta (C) of LDLR<sup>-/-</sup> mice reconstituted with WT (black circles), Mal1<sup>-/-</sup> (white circles), or aP2<sup>-/-</sup> (gray circles) bone marrow cells. Data from fast performance liquid chromatography analysis (B) are represented as the average (n=3 per group) percentage of total cholesterol for each fraction. Fractions 14 to 17 contained very-low-density lipoprotein (VLDL); fractions 18 to 24 contained IDL/low-density lipoprotein (LDL); and fractions 25 to 30 contain high-density lipoprotein (HDL). Note the differences (\**P*<0.05) between mice reconstituted with WT marrow vs mice transplanted with Mal1<sup>-/-</sup> or aP2<sup>-/-</sup> marrow determined by 1-way ANOVA.

generate LDLR<sup>-/-</sup> mice with hematopoietic cells from Mal1<sup>-/-</sup> mice (n=15), aP2<sup>-/-</sup> mice (n=15), or control WT mice (n=13). Four weeks posttransplantation, recipient mice were challenged with a Western diet for 8 weeks. There was a steady increase in body weight, with no differences between the groups (Figure 1A). No significant differences were found in serum total cholesterol and triglyceride levels between the groups either on chow or Western diet for 4 and 8 weeks (Table). Similarly, size exclusion chromatography analyses of plasma lipoproteins revealed no differences between mice reconstituted with WT, Mal1<sup>-/-</sup>, or aP2<sup>-/-</sup> marrow (Figure 1B). However, recipient mice receiving Mal1<sup>-/-</sup> or aP2<sup>-/-</sup> bone marrow cells had significantly reduced size (36% and

**Table. Total Serum Cholesterol and Triglyceride Levels in LDLR<sup>-/-</sup> Mice Reconstituted With WT, Mal1<sup>-/-</sup>, or aP2<sup>-/-</sup> Bone Marrow**

Type of Marrow Reconstituted	Serum Lipid	Baseline	4-W Western Diet	8-W Western Diet
WT cells (n=13)	Cholesterol	201±9	476±26	597±26
	Triglycerides	95±8	198±11	207±21
Mal1 <sup>-/-</sup> cells (n=14)	Cholesterol	208±3	433±16	584±18
	Triglycerides	91±5	194±16	228±14
aP2 <sup>-/-</sup> cells (n=15)	Cholesterol	202±5	472±15	601±26
	Triglycerides	97±5	177±12	209±12

Values are in mg/dL (mean±SEM). The number of recipient mice in each group is indicated by n. The differences were not statistically significant between the groups at either time point.

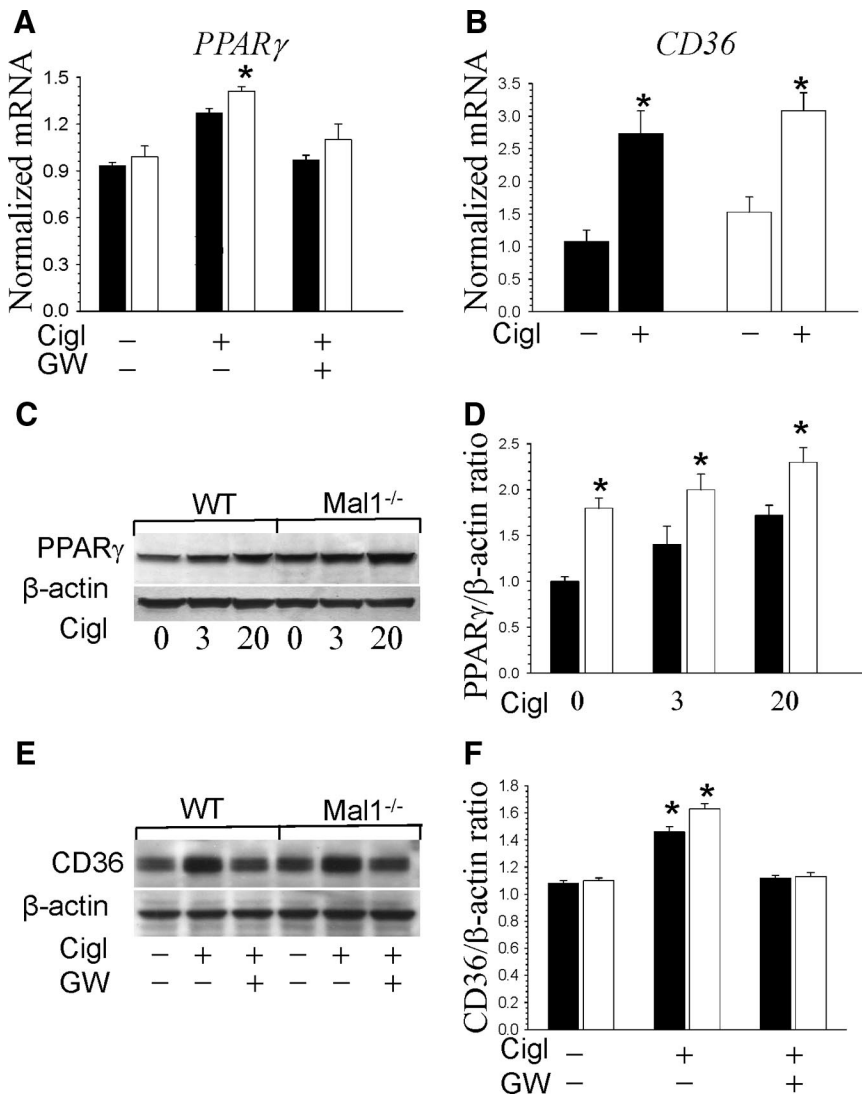
21%) of atherosclerotic lesions in the proximal aorta compared with mice transplanted with WT cells (Figure 1C).

### Mal1 Deficiency Increases PPAR $\gamma$ Activity in Macrophages

Recent studies have implicated enhanced PPAR $\gamma$  activity in aP2<sup>-/-</sup> macrophages as the mechanism responsible for altering expression of genes that regulate cholesterol homeostasis and inflammation resulting in inhibition of atherosclerosis.<sup>6</sup> Because Mal1 has many similarities to aP2 in both structure and function, we hypothesized that the antiatherogenic effects of Mal1 deficiency may result from a similar mechanism. To test this hypothesis, we isolated peritoneal macrophages from WT and Mal1<sup>-/-</sup> mice, and incubated them with Dulbecco's modified Eagle's medium containing 10% lipoprotein-deficient serum overnight. Then, cells were treated with fresh medium alone (control) or with a potent PPAR $\gamma$  agonist, ciglitazone, with or without the selective PPAR $\gamma$  antagonist GW9662. Real-time PCR analysis indicated that PPAR $\gamma$  activation increased expression of the PPAR $\gamma$  gene significantly higher in Mal1<sup>-/-</sup> macrophages than in WT cells, and these effects were completely reversed by addition of the PPAR $\gamma$  antagonist (Figure 2A). Similarly, the ligand treatment significantly (1.5-fold) increased expression of the CD36 mRNA in both WT and Mal1<sup>-/-</sup> macrophages (Figure 2B). There was a similar trend that was not statistically significant for an increase in expression of the ABCA1 and ABCG1 genes (data not shown), which are regulated indirectly by PPAR $\gamma$  through LXR $\alpha$ . Interestingly, the expression of the CD36, ABCA1, and ABCG1 genes was significantly increased in Mal1<sup>-/-</sup> macrophages compared with WT cells (Supplemental Figure I, available online at <http://atvb.ahajournals.org>) when they were loaded with OxLDL or free cholesterol by incubating them with AcLDL together with the ACAT inhibitor Sandoz 58035, as described.<sup>17</sup> These data strongly suggest that the PPAR $\gamma$  pathway is upregulated in Mal1-null macrophages.

Next, WT and Mal1<sup>-/-</sup> macrophages were incubated with increasing doses of ciglitazone. Then proteins were extracted from the cells and analyzed by Western blot. The ligand treatment significantly increased PPAR $\gamma$  protein expression in both types of cells but was always higher in Mal1<sup>-/-</sup> macrophages than WT cells (Figure 2C and 2D). Similarly,





**Figure 2.** Expression of PPAR $\gamma$  (A) and CD36 (B) genes and PPAR $\gamma$  (C and D) and CD36 (E and F) protein expression levels in WT and Mal1<sup>-/-</sup> macrophages treated with the PPAR $\gamma$  agonist ciglitazone (A to E) alone or together with the specific PPAR $\gamma$  antagonist GW9662 (A). A and B, Thioglycollate-elicited WT (■) and Mal1<sup>-/-</sup> (□) macrophages were treated with lipid-free Dulbecco's modified Eagle's medium containing the PPAR $\gamma$  agonist ciglitazone (Cigl, 15  $\mu$ M/L), alone or together with the specific PPAR $\gamma$  antagonist GW9662 (GW, 10  $\mu$ M/L), at 37°C for 24 hours. Then RNA was extracted from the macrophages and analyzed by real-time PCR. Graphs represent data (mean  $\pm$  SEM) of analysis the same number (n=3) of mice per group (\* $P$ <0.05 between untreated and treated with the ciglitazone macrophages of the same group). C to F, WT and Mal1<sup>-/-</sup> macrophages were treated with ciglitazone (15  $\mu$ M/L), alone or together with GW9662 (30  $\mu$ M/L), for 24 hours. Extracted proteins (50  $\mu$ g/well) were resolved and analyzed by Western blot. The data of PPAR $\gamma$ / $\beta$ -actin and CD36/ $\beta$ -actin ratios are presented as average (mean  $\pm$  SEM) of the assay of 3 separate experiments. \*Differences between the groups with the same dose (D,  $P$ <0.05) or between control cells and treated with ciglitazone macrophages (F,  $P$ <0.001).

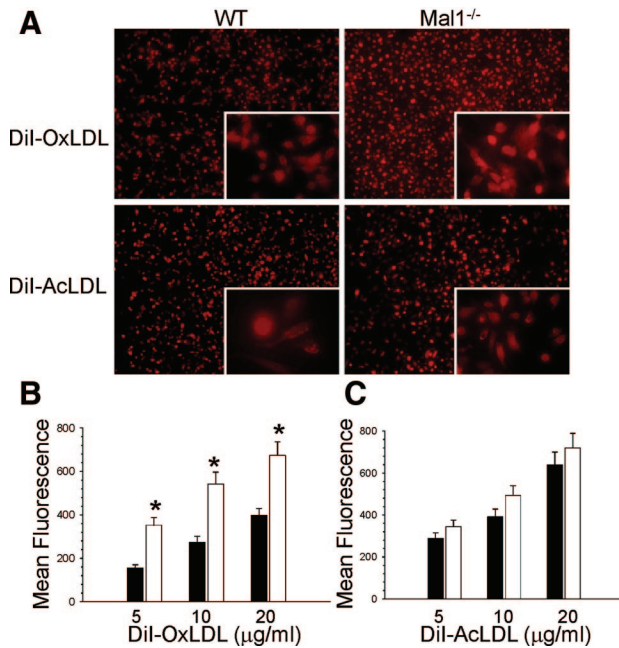
the ciglitazone treatment significantly (1.5-fold) increased CD36 protein expression levels in both WT and Mal1<sup>-/-</sup> macrophages (Figure 2E and 2F). Then, to verify the role of PPAR $\gamma$  in mediating the increase in CD36, we made use of the PPAR $\gamma$  antagonist GW9662, which covalently modifies a cysteine residue of PPAR $\gamma$ , resulting in complete loss of the ligand-binding ability of PPAR $\gamma$ .<sup>19</sup> Interestingly, treatment of cells with the PPAR $\gamma$  antagonist GW9662 in conjunction with ciglitazone abolished the increase of CD36 expression in both types of macrophages (Figure 2E and 2F).

Finally, we examined whether activation of scavenger receptor CD36, which is directly regulated by PPAR $\gamma$ , has an effect on uptake of modified low-density lipoprotein. WT and Mal1<sup>-/-</sup> macrophages were incubated with 3,3'-diiododecylindocarbocyanine-labeled AcLDL and 3,3'-diiododecylindocarbocyanine-labeled OxLDL for 2 hours and then analyzed visually and by flow cytometry. Compared with WT cells, Mal1<sup>-/-</sup> macrophages displayed increased uptake of OxLDL (Figure 3A). FACS analysis demonstrated that Mal1<sup>-/-</sup> macrophages had increased OxLDL uptake (69% to 125%) but only slightly increased AcLDL uptake (13% to 26%; Figure 3C). Taken together, these data indicate that Mal1 deficiency acti-

vates the PPAR $\gamma$  pathway in macrophages, enhancing expression of the PPAR $\gamma$ -regulated gene CD36, and results in upregulation of CD36-mediated uptake of OxLDL.

### Mal1 Deficiency Increases Akt Phosphorylation and Suppresses COX2 and Interleukin 6 Gene Expression in Macrophages

Because FABP expression may change Akt activity in different types of cells,<sup>9</sup> we compared Mal1<sup>-/-</sup> and WT peritoneal macrophages in their response to a lipotoxic factor, palmitic acid (0.5 mmol/L) complexed with BSA, used to induce endoplasmic reticulum stress-related signaling.<sup>16</sup> Mal1<sup>-/-</sup> cells had significantly increased levels of Akt activation (Figure 4A; p-Akt/ $\beta$ -actin ratios were 2.2, 1.6, and 1.7 versus 1.0, 1.4, and 1.2, respectively, in WT cells), a survival factor that is capable of modulating inflammatory pathways, including NF- $\kappa$ B. In contrast, the expression levels of Akt and  $\beta$ -actin were not significantly different in these 2 types of cells (Figure 4A; Akt/ $\beta$ -actin ratios were 1.0 to 1.2 and 1.0 to 1.1, respectively). Similarly, treatment with palmitic acid (0.5 mmol/L) complexed with BSA resulted in significantly less I $\kappa$ B $\alpha$  protein in Mal1<sup>-/-</sup> macrophages compared with



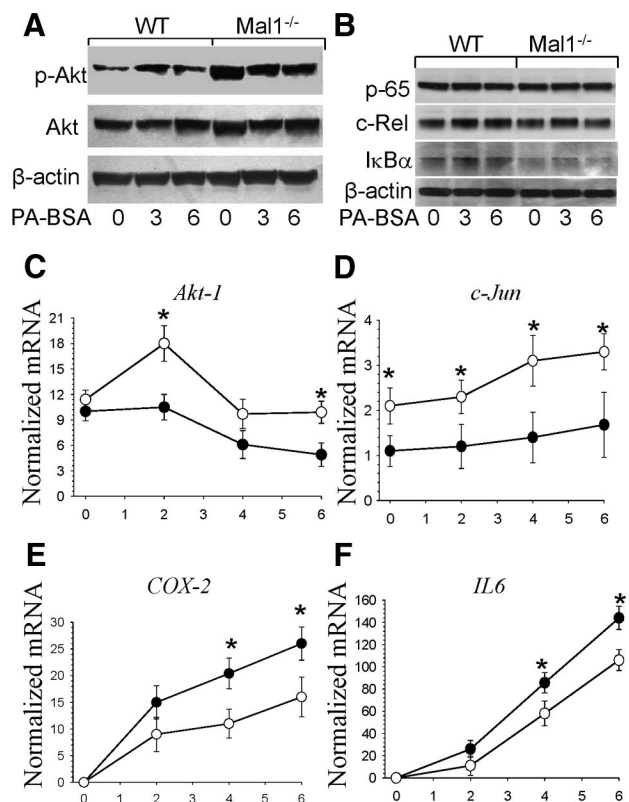
**Figure 3.** Visualization (A) and quantified uptake of human 3,3'-diiododipalmitoylcardiolipin (Dil)-OxLDL (B) or Dil-AcLDL (C) by macrophages from WT (■) and Mal1<sup>-/-</sup> (□) mice. A, Peritoneal macrophages were incubated with human Dil-OxLDL (10 μg/mL) or Dil-AcLDL (10 μg/mL) for 2 hours and examined under the microscope (Olympus BX-40) (magnification ×20; inset magnification ×60). B and C, Peritoneal macrophages were incubated with the indicated doses of human Dil-OxLDL or Dil-AcLDL for 1 hour and analyzed by FACS.

WT cells (Figure 4B, 0.73, 0.81, and 0.53 versus 1.0, 1.35, and 1.7, respectively). The levels of other NF-κB-related proteins, such as p-65 and c-Rel, were not significantly different in these cells (Figure 4B). Interestingly, Mal1<sup>-/-</sup> macrophages had significantly higher levels of basal and lipopolysaccharide-mediated (50 ng/mL) Akt-1 and c-Jun gene expression than WT cells (Figure 4C and 4D). In contrast, lipopolysaccharide induced less COX2 and interleukin 6 (IL6) gene expression in Mal1<sup>-/-</sup> macrophages compared with WT cells (Figure 4E and 4F). These data indicate that Mal1 deficiency increases Akt activity and suppresses IκBα protein, COX2, and IL6 gene expression levels in macrophages.

### Mal1 Deficiency Decreases Macrophage Cell Numbers in Atherosclerotic Lesions and Suppresses CCR2 Expression by Blood Monocytes

To test whether macrophage Mal1 deficiency affects cell density in atherosclerotic lesions, we analyzed the number of nuclei (stained by 4',6-diamidino-2-phenylindole) in the macrophage (MOMA-2 positive) area of atherosclerotic lesions in the proximal aorta. Compared with control LDLR<sup>-/-</sup> mice transplanted with WT marrow (Figure 5A to 5C), LDLR<sup>-/-</sup> mice reconstituted with Mal1<sup>-/-</sup> bone marrow (Figure 5D to 5F) had significantly (36%) lower numbers of macrophages in the lesion area (Figure 5G). These data suggest that Mal1<sup>-/-</sup> macrophages undergo decreased recruitment to atherosclerotic lesions.

Next, we lethally irradiated and transplanted male 10-week-old LDLR<sup>-/-</sup> mice with Mal1<sup>-/-</sup> (n=5) or WT

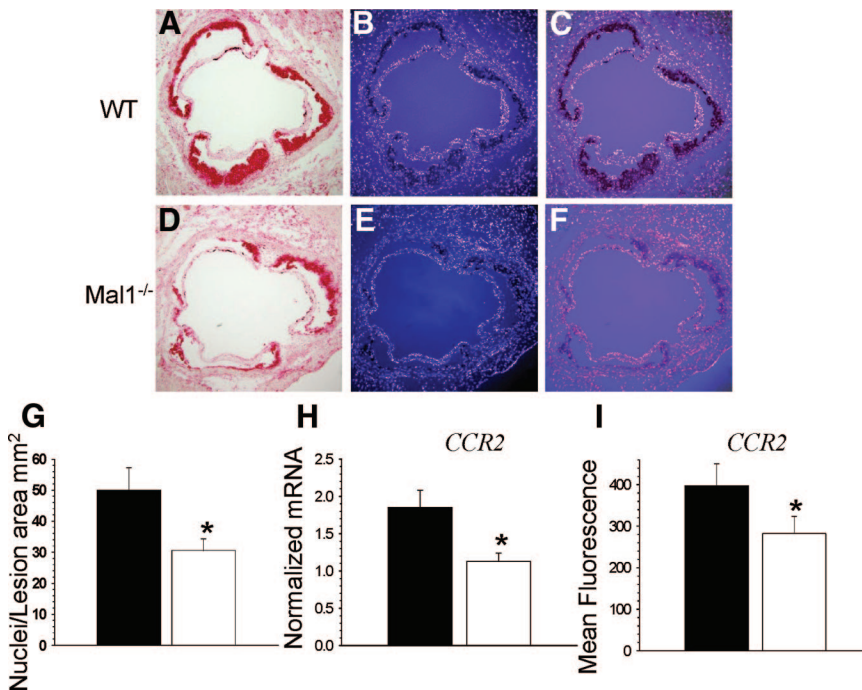


**Figure 4.** Treatment with palmitic acid complexed with BSA (A and B) or lipopolysaccharide (C to F) significantly increased activity of Akt and slightly suppressed NF-κB-related protein and genes in Mal1-deficient macrophages. A and B, Macrophages were incubated with medium alone or with PA-BSA (0.5 mmol/L) for 3 and 6 hours. Extracted proteins were resolved (100 μg/well) and analyzed by Western blot using antibodies to Akt, p-Akt, or β-actin (A), or extracted proteins (20 μg/well) were analyzed by Western blot using antibodies to p-65, c-Rel, IκBα, or β-actin (B). C to F, Peritoneal macrophages from WT (●) and Mal1<sup>-/-</sup> (○) mice were treated with lipopolysaccharide (50 ng/mL) for the indicated time periods, and the expression of the Akt-1 (C), c-Jun (D), COX2 (E), and IL6 (F) genes was analyzed by real-time PCR.

(n=5) marrow. Eight weeks later, recipient mice were fed with the Western diet for 12 weeks. Blood monocytes were isolated from the recipient mice, and CCR2 gene and protein levels were analyzed by real-time PCR and FACS. Real-time PCR demonstrated that Mal1<sup>-/-</sup> monocytes expressed significantly lower (64%) levels of levels of CCR2 mRNA compared with WT cells (Figure 5H). Compared with control WT control monocytes, the Mal1<sup>-/-</sup> monocytes also had suppressed (28%) levels of CCR2 protein expression as detected by FACS (Figure 5I). Similar suppression of CCR2 gene and protein expression levels was noted in Mal1<sup>-/-</sup>/apoE<sup>-/-</sup> monocytes compared with apoE<sup>-/-</sup> cells (Supplemental Figure II). Taken together, the results indicate that upregulation of the PPARγ pathway in Mal1<sup>-/-</sup> mice suppresses CCR2 expression in blood monocytes, and this likely affects their recruitment to atherosclerotic lesions.

### Discussion

The adipocyte/macrophage FABPs aP2 and Mal1 link features of the metabolic syndrome, including insulin resistance



**Figure 5.** Mal1 deficiency decreased the number of nuclei in the MOMA-2+ area of atherosclerotic lesions (A to G) and suppressed CCR2 gene (H) and protein (I) expression levels in blood monocytes. A to F, Serial sections from the proximal aorta of LDLR<sup>-/-</sup> mice reconstituted with WT (A to C) and Mal1<sup>-/-</sup> (D to F) marrow and fed the Western diet for 12 weeks. Sections were stained with antibodies to the mouse macrophage MOMA-2 (A and D) and the nuclear stain 4',6-diamidino-2-phenylindole (B and E). After merging of the images, the number of nuclei was analyzed in MOMA-2-positive area. Note number of nuclei per standard lesion area (G; mean±SEM; \*P<0.05 between mice with WT and Mal1<sup>-/-</sup> marrow). G to I, Blood monocytes were isolated from WT→LDLR<sup>-/-</sup> (■) and Mal1<sup>-/-</sup>→LDLR<sup>-/-</sup> (□) mice on the Western diet. CCR2 gene (H) and protein (I) expression levels were analyzed by real-time PCR and FACS. Graphs represent data (Mean±SEM) analysis of the same number (n=3) mice per group (\*P<0.05 between WT and Mal1<sup>-/-</sup> cells).

and atherosclerosis.<sup>2</sup> Studies with aP2<sup>-/-</sup> or aP2<sup>-/-</sup>/Mal1<sup>-/-</sup> mice have shown that the elimination of these proteins in total body or exclusively in hematopoietic cells significantly suppresses atherosclerotic lesion formation in apoE<sup>-/-</sup> mice.<sup>3-5</sup> Remarkably, aP2 and Mal1 have additive effects with regard to insulin sensitivity, as aP2<sup>-/-</sup> mice show improved insulin sensitivity only in the setting of dietary or genetic obesity, whereas aP2<sup>-/-</sup>/Mal1<sup>-/-</sup> mice show improved insulin sensitivity on the apoE-deficient background even when lean and on a normal chow diet.<sup>4,5,13</sup> Expression of aP2 and Mal1 by both adipocytes and macrophages contributes to insulin resistance.<sup>20</sup> However, macrophage aP2 expression promotes atherosclerosis independently of its impact on insulin sensitivity.<sup>3</sup> Because both proteins, aP2 and Mal1, share a high degree of homology and are expressed by macrophages in similar proportions,<sup>5</sup> we examined the hypothesis that macrophage Mal1 expression influences the development of atherosclerosis. Here we demonstrate that Mal1 deficiency in hematopoietic cells significantly inhibits (36%) early atherosclerotic lesion formation in LDLR<sup>-/-</sup> mice compared with control mice reconstituted with WT bone marrow. This effect is not mediated by differences in serum lipids levels or lipoprotein distributions.

Previous studies have shown that macrophage aP2 deficiency significantly enhances PPAR $\gamma$  activity and suppresses atherosclerosis formation.<sup>6</sup> Therefore, to examine mechanisms underlying the impact of macrophage Mal1 expression on atherogenesis, we analyzed PPAR $\gamma$  gene and protein expression levels in peritoneal macrophages isolated from Mal1<sup>-/-</sup> and WT mice. We demonstrated that treatment with the PPAR $\gamma$  agonist ciglitazone increased PPAR $\gamma$  gene and protein expression more in Mal1<sup>-/-</sup> macrophages than in WT cells, and the effect on PPAR $\gamma$  gene expression was reversed by the addition of a PPAR $\gamma$  antagonist (Figure 2A). In addition, we showed in a dose-response study with ciglitazone that PPAR $\gamma$  protein expression increased to a greater extent in Mal1<sup>-/-</sup> macrophages than in WT cells (Figure 2C and 2D). Our results suggest an interesting possibility that the promoter of the PPAR $\gamma$  gene may contain functional PPAR $\gamma$  response element (PPRE) sites. Interestingly, a gene database search of the mouse and human PPAR $\gamma$  gene promoters revealed 3 potential PPRE sites. The site with the highest score (AGGGCAAAGGCCT) is 100% conserved between human and mouse, has 10 of 13 (76.9%) identity with the consensus PPRE sequence, and reveals high similarity with known functional PPREs in PPAR $\gamma$  target genes. (Supplemental Figure III). We also demonstrated that expression of CD36, which is directly regulated by PPAR $\gamma$ , was increased to a greater extent in Mal1<sup>-/-</sup> macrophages than in WT cells (Figure 3B), and the ciglitazone-related increase in CD36 protein expression was abolished by the PPAR $\gamma$  antagonist GW9662 (Figure 2E and 2F). There was also a trend for an increase in expression of ABCA1 and ABCG1 gene expression in ciglitazone-treated Mal1<sup>-/-</sup> macrophages, but it was not statistically significant (data not shown). It is important to note that PPAR $\gamma$  regulates ABCA1 through LXR $\alpha$ , and our failure to see a significant increase in ABCA1 may be due to the conditions of the experiment in that the cells were not loaded with cholesterol. Indeed, Mal1<sup>-/-</sup> macrophages showed increased expression of CD36, ABCA1, and ABCG1 in response to OxLDL and free cholesterol loading (Supplemental Figure I). These findings are consistent with earlier studies reporting that FABPs bind PPAR ligands and that FABP overexpression inhibits lipid-mediated signaling to the PPARs.<sup>21</sup> A similar PPAR $\gamma$ -active phenotype has been described in aP2<sup>-/-</sup> macrophages, and it was associated with an antiinflammatory status.<sup>6</sup> Previous studies have demonstrated that activation of the PPAR $\gamma$ -LXR $\alpha$  pathway reciprocally regulates inflammation and lipid metabolism,<sup>22</sup> stimulating genes involved in cholesterol homeostasis<sup>23</sup> and antagonizing

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genes encoding inflammatory proteins.<sup>8</sup> Recent studies have suggested that free cholesterol accumulation may induce a proinflammatory phenotype in macrophages.<sup>24</sup> Indeed, the increased cellular free cholesterol and lipid raft contents in ABCA1<sup>-/-</sup> macrophages enhance expression of proinflammatory cytokines and activation of the NF- $\kappa$ B pathway.<sup>25</sup> Taken together, these data indicate that Mal1 deficiency activates the PPAR $\gamma$  pathway in macrophages, protecting them against proinflammatory and proatherogenic changes.

We also noted that Mal1 deficiency significantly increased basal and stimulated Akt activity in macrophages. Interestingly, a similar increase of basal and insulin-stimulated phosphorylation of Akt has been noted in adipose and muscle tissues of aP2<sup>-/-</sup>/Mal1<sup>-/-</sup> mice.<sup>9</sup> The Akt activation is higher in the presence of shorter chain (12:0 and 14:0) fatty acids and strongly inhibited in the presence of longer chain (16:0 and 18:0) fatty acids.<sup>9</sup> Akt signaling promotes cell survival but also modulates inflammatory responses and stimulates transport and metabolism of glucose and amino acids.<sup>26</sup> It is important to note that macrophages constitutively express p-Akt, and inhibition of the pathway significantly accelerates their apoptosis.<sup>11</sup> Consistent with these data, Akt1-null macrophages are more susceptible to apoptotic stimuli.<sup>27</sup>

Macrophage aP2 deficiency has been shown to reduce the activity of I $\kappa$ B, which may, at least in part, underlie the alterations in cytokine expression.<sup>6</sup> Similarly, we found that Mal1 deficiency suppressed COX2, IL6 mRNA, and I $\kappa$ B protein expression in macrophages, although to a lesser degree than is seen in aP2<sup>-/-</sup> macrophages.<sup>6</sup> Next, we demonstrated that LDLR<sup>-/-</sup> mice reconstituted with Mal1<sup>-/-</sup> bone marrow had decreased numbers of macrophages in their atherosclerotic lesions compared with control mice transplanted with WT bone marrow (Figure 5A to 5G). This strongly supports the hypothesis that the recruitment of Mal1<sup>-/-</sup> monocytes to atherosclerotic lesions may be impaired, leading to diminished cell numbers in the atherosclerotic lesions. CCR2 is known as a receptor for monocyte chemoattractant protein 1, which plays pivotal roles in immune responses and atherosclerosis.<sup>28</sup> CCR2 is necessary for efficient monocyte recruitment from the blood to inflamed tissue and to atherosclerotic lesions.<sup>28</sup> A recent study has identified PPAR $\gamma$  as a critical signaling molecule in determining macrophage phenotype in vitro, and treatment with a PPAR $\gamma$  agonist enhances the antiinflammatory properties of macrophages.<sup>29</sup> In this regard, PPAR $\gamma$  activation by OxLDL inhibits CCR2 expression in human and mouse monocytes.<sup>30</sup> In contrast, macrophage-specific deficiency in PPAR $\gamma$  significantly accelerates CCR2 expression and atherosclerosis in LDLR<sup>-/-</sup> mice.<sup>15</sup> These findings suggest the hypothesis that reduced expression of CCR2 by Mal1<sup>-/-</sup> monocytes may contribute to the reduction in atherosclerosis in the Mal1<sup>-/-</sup>→LDLR<sup>-/-</sup> mice. We did not see an impact of monocyte chemoattractant protein 1 on macrophage migration in an in vitro migration assay comparing unstimulated Mal1<sup>-/-</sup> and WT peritoneal macrophages (data not shown). However, these results do not rule out an important role for reduced CCR2 expression by Mal1<sup>-/-</sup> monocytes in reducing recruitment to atherosclerotic lesions in vivo. We provide evidence from Mal1<sup>-/-</sup>→LDLR<sup>-/-</sup> mice fed a high-fat diet that

Mal1<sup>-/-</sup> monocyte CCR2 gene and protein expression levels are reduced in vivo compared with WT cells from control Mal1<sup>+/+</sup>→LDLR<sup>-/-</sup> mice. The relevance of this finding is supported by our data showing that monocyte deficiency of Mal1<sup>-/-</sup> reduced CCR2 expression in vivo in a second genetic model of atherosclerosis, apoE<sup>-/-</sup> mice (Supplemental Figure II). Although the impact of CCR2 expression on atherogenesis is not limited to its role in recruitment,<sup>31</sup> we believe that our findings of reduced expression of CCR2 in Mal1-deficient monocytes, coupled with the in vivo evidence that the atherosclerotic lesions of Mal1<sup>-/-</sup>→LDLR<sup>-/-</sup> mice have reduced numbers of macrophages, support the hypothesis that reduced CCR2 expression by Mal1 monocytes contributes to the reduced atherosclerosis in the Mal1<sup>-/-</sup>→LDLR<sup>-/-</sup> mice, likely because of an impact on recruitment. Together, these data show that Mal1 expression regulates inflammatory activity in macrophages and likely affects monocyte recruitment to atherosclerotic lesions.

In conclusion, our results demonstrate that macrophage Mal1 plays an important role in early atherosclerosis. As a key regulator of PPAR $\gamma$  activity and inflammatory responses in macrophages, Mal1 modulates monocyte recruitment and the development of atherosclerotic lesions. These findings support macrophage Mal1 as a potential therapeutic target for the prevention of atherosclerosis. The potential relevance of these findings is supported by previous studies demonstrating that a small-molecule inhibitor of aP2 is able to retard the development of diabetes and atherosclerosis in murine models.<sup>32</sup>

## Sources of Funding

This work was supported by National Institutes of Health Grants HL65405, HL105375, HL53989, DK064360, ADA(7-02-RA-38), 0555323B, HL57986, and DK59637 (Lipid, Lipoprotein and Atherosclerosis Core of the Vanderbilt Mouse Metabolic Phenotype Centers).

## Disclosures

None.

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# Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

## **Macrophage Mal1 Deficiency Suppresses Atherosclerosis in Low-Density Lipoprotein Receptor–Null Mice by Activating Peroxisome Proliferator-Activated Receptor- $\gamma$ -Regulated Genes**

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*Arterioscler Thromb Vasc Biol.* 2011;31:1283-1290; originally published online April 7, 2011;  
doi: 10.1161/ATVBAHA.111.225839

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272  
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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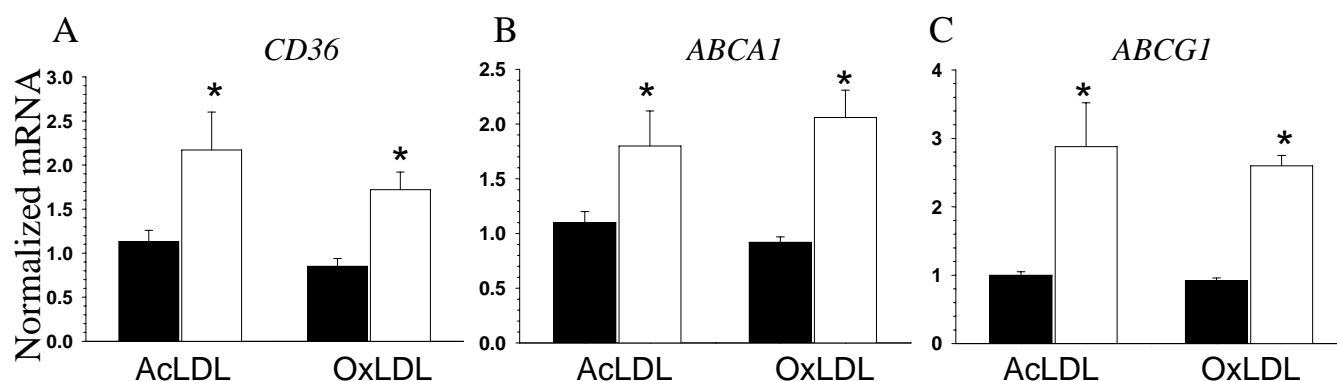
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## Supplement Methods

*Computer-based search for putative PPRE in PPAR-gamma promoter.* To identify putative PPAR-responsive elements (PPREs) in the PPAR-gamma promoter, internet-based (<http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home>) Transcriptional Regulatory Element Database and Transcription Element Search System (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>) were applied to search for the motif in the 2,000 bps of mouse and human PPAR-gamma promoter by PPAR algorithm (JASPAR MA0066)<sup>1</sup> as we described previously<sup>2</sup>.

## Supplement Data



**Figure I. Expression of PPAR $\gamma$ -related genes in WT and Mal1<sup>-/-</sup> peritoneal macrophages treated with AcLDL or OxLDL for 48 hours.**

Thioglycollate-elicited macrophages were isolated from WT and Mal1<sup>-/-</sup> mice and loaded with OxLDL (100mg/ml) or free cholesterol by incubating them with AcLDL (100mg/ml) plus an ACAT inhibitor, Sandoz 58035 (10mg/ml), for 24 hours. Total RNA was extracted from cells and analyzed by real-time PCR.

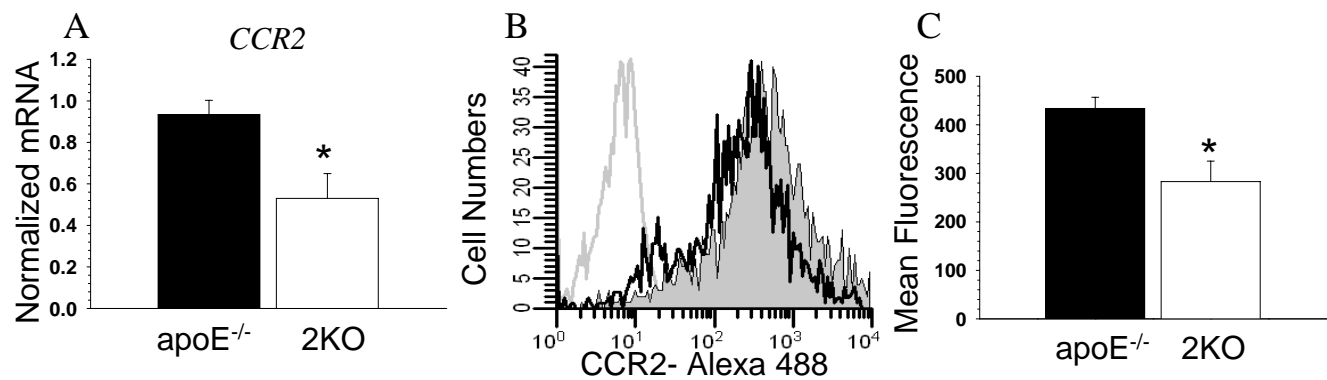


Figure II. **CCR2 gene and protein expression levels in WT(■) and Mal1<sup>-/-</sup>(□) peritoneal macrophages.**

Blood monocytes were isolated from Mal1<sup>-/-</sup>/apoE<sup>-/-</sup> (n=5) and apoE<sup>-/-</sup> (n=5) mice fed the Western diet for more than two weeks. CCR2 gene and protein expression levels were analyzed by real-time PCR and by FACS. Note ApoE<sup>-/-</sup> macrophages expressed higher levels of CCR2 gene (A) and protein (B,C). CCR2 protein expression levels in unstained (gray line) or stained apoE<sup>-/-</sup> monocytes (filled with gray color) versus Mal1<sup>-/-</sup>/apoE<sup>-/-</sup> cells (bold black line). Graphs represent data (Mean ± SEM; \*p<0.05 between these groups).

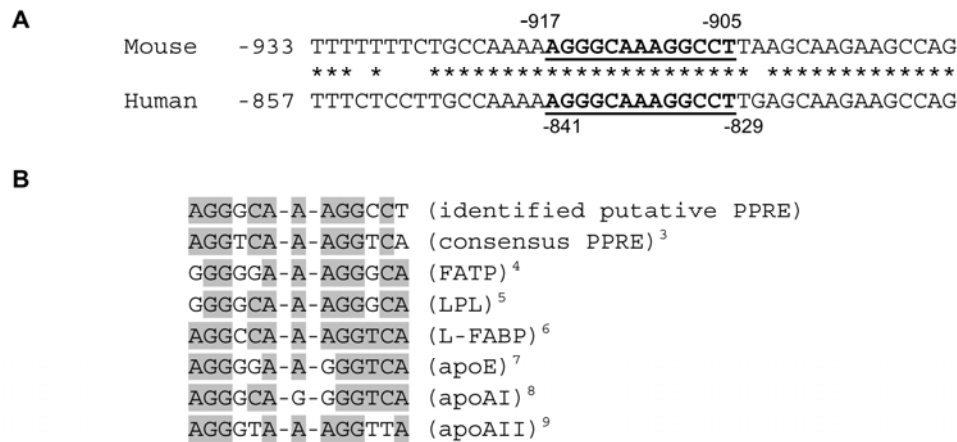


Figure III. **Identification of a putative PPAR $\gamma$  responsive element (PPRE) in the mouse and human PPAR-gamma genes.**

**A:** A computer-based program search yielded three candidate sequences from low to high probability score (0.14 to 4.09 at a cutoff score of 0). We further analyzed these sequences by comparing the conservation between the human and mouse species and by comparing with consensus PPRE and known functional PPREs in PPAR-gamma target genes. We found the site with the highest score



(AGGGCAAAGGCCT) is located between -917 to -905 in mouse PPAR-gamma promoter (NM\_011146) and -841 to -829 in human PPAR-gamma promoter (NM\_005037) and it is 100% conserved between human and mouse and has 10/13 (76.9%) of identity with consensus PPRE sequence. The identified putative PPRE is bold and underlined in the mouse and human PPAR-gamma promoters. Comparison of conservation of the putative PPRE between mouse and human species is 100%.

- B:** Comparison of the putative PPRE with consensus and known PPRES in PPAR-gamma target genes reveals high similarity with known functional PPRES in PPAR-gamma target genes.

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